Type 1 diabetes impairs the mobilisation of highly-differentiated CD8+T cells during a single bout of acute exercise

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ABSTRACT

Type 1 diabetes (T1D) is a T cell mediated autoimmune disease that targets and destroys insulin-secreting pancreatic beta cells. Beta cell specific T cells are highly differentiated and show evidence of previous antigen exposure. Exercise-induced mobilisation of highly-differentiated $CD8^+$ T cells facilitates immune surveillance and regulation. We aimed to explore exercise-induced T cell mobilisation in T1D. In this study, we compared the effects of a single bout of vigorous intensity exercise on T cell mobilisation in T1D and control participants. N=12 T1D (mean age 33.2yrs, predicted VO_2 max 32.2 mL/(kg·min), BMI 25.3Kg/m2) and N=12 control (mean age 29.4yrs, predicted VO_2 max 38.5mL(kg.min), BMI 23.7Kg/m2) male participants completed a 30-minute bout of cycling at 80% predicted VO_2 max in a fasted state. Peripheral blood was collected at baseline, immediately post-exercise, and 1 hour post-exercise. Exercise-induced mobilisation was observed for T cells in both T1D and control groups. Total $CD8^+$ T cells mobilised to a similar extent in T1D (42.7%; p=0.016) and controls (39.7%; p=0.001). CD8 effector memory $CD45RA^+$ (EMRA) subset were the only T cell lineage subset to be significantly mobilised in both groups though the percentage increase of $CD8^+$ EMRA was blunted in T1D (T1D (26.5%) p=0.004, control (66.1%) p=0.010). Further phenotyping of these subsets revealed that the blunting was most evident in $CD8^+$ EMRA that expressed adhesion (CD11b: T1D 37.70%, Control 91.48%) and activation markers (CD69: T1D 29.87%, Control 161.43%), and appeared to be the most differentiated ($CD27^-CD28^-$: T1D 7.12%, Control 113.76%). $CD4^+$ T cells mobilised during vigorous intensity exercise in controls (p=0.001), but not in T1D. The blunted mobilisation response of particular T cell subsets was not due to CMV serostatus or apparent differences in exertion during the exercise bout as defined by heart rate and RPE. Predicted VO_2 max showed a trend to be lower in the T1D group than the control group but is unlikely to contribute to this blunted response. We postulate the reasons for a blunted mobilisation of differentiated $CD8^+$ EMRA cells includes differences in blood glucose, adrenaline receptor density, and sequestration of T cells in the pancreas of T1D participants. In conclusion, mobilisation of $CD8^+$ EMRA and $CD4^+$ subsets T cells is decreased in people with T1D during acute exercise.

Key words: Exercise, Physical activity, Type 1 Diabetes, Immunity, T cells

INTRODUCTION

Type 1 Diabetes (T1D) is an autoimmune disorder characterised by T cell mediated destruction of insulin secreting pancreatic beta cells. Peripheral T cells are mobilised by acute exercise in health, and exercise may also modulate the number and function of these cells. We therefore wished to explore the effect of exercise on T cell mobilisation in people with T1D.

T cells in T1D

Beta cell specific T cell subsets are mainly comprised of late differentiated memory T cells that show evidence of previous antigen exposure [1-3]. Indeed, circulating islet specific $CD8^+$ T cells have been detected at similar frequencies in both T1D and non-diabetic cohorts [4]. However, antigen-experienced islet reactive $CD8^+$ T cells ($ZnT8_{186–194}$ $multimer^+$) were found sequestered in the pancreas of T1D donors, but not non-diabetic donors [4]. Islet resident $CD8^+$ T cells in the human pancreas also express CD11b, CD69, and CD103, which are markers of tissue resident memory cells [5-7]. In particular, elevated highly differentiated $CD8^+$ effector memory T cells re-expressing CD45RA (EMRA) have been found in T1D [1, 2, 8].

T cell differentiation

Re-expression of CD45RA on fully differentiated effector memory (EM) T cells (EMRA) is the final stage of T cell differentiation in the T cell lineage pathway [9] (Figure 1), which gives rise to a range of phenotypically and functionally diverse T cell subsets. This re-expression of CD45RA leads to a more stable resting memory T cell pool that can respond to recall antigens [10, 11]. Differentiation status of EMRA subsets can be identified in a number of ways; cell surface expression of markers such as CD26, CD57, and killer cell lectin-like receptor subfamily G member 1 (KLRG1) [1, 2, 8]; lack of CD27 and CD28 expression [12, 13]; and evidence of shortened telomeres indicative of successive rounds of cell division [2, 8]. Although conventionally EMRA are $CD27^-CD28^-$, there is evidence of further subdivisions of differentiation based on the differential expression of these markers [14].

Pedro Romero et al., 2007 identified small subpopulations of $CD27^{-}CD28^{+}$ and $CD27^{+}CD28^{-}$ EMRA [14]. Nonetheless, $CD27^{+}CD28^{+}$ and $CD27^{-}CD28^{-}$ EMRA make up the larger proportion of EMRA subpopulations [14, 15].

Towards the beginning of the linear differentiation pathway, $CD8^{+}$ T cells are released into the circulation as naïve T cells which are characterised by their cell surface co-expression of CD45RA, CCR7, CD27 and CD28 [16-21]. CD45, the leukocyte common antigen, has multiple isoforms which are differentially expressed based on maturation status [22]. Central memory (CM) cells are formed ($CD45RA^{-}CCR7^{+}$) following antigen experience and loss of CD45RA [18, 21]. Memory cells lose the expression of the high molecular weight CD45RA isoform and gain expression of the low molecular CD45RO isoform following activation [23]. Following antigen presentation, CM T cells can rapidly differentiate into T cells with effector functions [20]. CCR7 is down-regulated as CM T cells become specialised, antigen specific effector T cells. CM T cells progress to effector memory (EM) T cells through three progressively differentiated subpopulations defined based on their expression of CD27 and CD28: early differentiated (ED) ($CD27^{+}CD28^{+}$), early-like differentiated (ELD) ($CD27^{-}CD28^{+}$), and intermediate differentiated (ID) ($CD27^{+}CD28^{-}$). CD27 and CD28 diminish in a stepwise fashion following successive rounds of differentiation in response to antigen stimulus, corresponding with an increase in cytotoxic functions [12, 13, 24]. Following the loss of both CD27 and CD28, ID T cells become fully differentiated EM T cells ($CD27^{-}CD28^{-}$) [24, 25].

Amidst these earlier stages of differentiation exists a dynamic population $CD8^{+}$ T cells which have recently been implicated in T1D [2, 3]. Stem cell like memory T cells (T_{SCM}) ($CD27^{+}CD45RO^{-}CD95^{+}CCR7^{+}$) express naïve surface markers (CD45RA, CCR7) and are capable of extensive proliferation and self-renewal, similar to hematopoietic stem cells [26-28]. However, T_{SCM} also have the ability to rapidly acquire effector functions upon stimulation and respond to recall antigens, analogous to effector memory subsets [28, 29]. Beta cell specific T cell populations in the peripheral blood have been identified with this phenotype in T1D, indicating a role for T_{SCM} in the pathogenesis of the disease [2, 3].

In addition to progressive differentiation of $CD8^{+}$ T cell lineage subsets, $CD4^{+}$ T cells are characterised in a similar manner (Figure 1). However, in addition to this phenotypic approach, $CD4^{+}$ T cells are also defined based on their differentiation into T-helper (Th) ($CD45RO^{+}CD127^{hi}CD25^{low}$) and T-Regulatory (TRegs) ($CD4^{+}CD127^{low}CD25^{+}$) subsets [17,

30]. The T-helper subpopulations are distinguished by chemokine receptor expression and cytokine secretion patterns [17, 31-33]. These include type 1 helper (Th1) cells [17, 34, 35], type 2 helper (Th2) [17, 34], Th9 [36], Th17 [17, 37-39], and Th22 cells [36]. $CD4^+$ TReg subpopulations consist of naïve (naTRegs) and memory TRegs (mTRegs). As described for lineage T cell subsets, naTRegs express CD45RA whereas mTRegs lose CD45RA and acquire CD45RO upon antigen exposure [18, 40]. mTRegs are an activated antigen primed subset of $CD4^+$ TRegs [41, 42]. mTREGs express TIGIT, a coinhibitory molecule [43], which supress pro-inflammatory Th1 and Th17 cells, but promote Th2 cell responses and therefore support an anti-inflammatory environment [43]. In T1D, there is strong evidence to suggest that the balance between T-helper and TRegs is disrupted. $CD4^+$ Th differentiation is skewed towards increased pro-inflammatory Th1 and Th17 cells [44, 45]. Whereas $CD4^+$ TReg function, and potentially TReg frequency, are compromised in T1D [46]. This increases immune dysregulation already upheld by memory $CD8^+$ T cell subsets.

Acute exercise and T cell mobilisation

To date, the effects of acute exercise on T cells, including those implicated in T1D pathogenesis, outlined above, have not been investigated. In healthy individuals, acute exercise induces a significant increase in the T cell frequency, proportional to exercise intensity, in the peripheral blood immediately following exercise.

The largest mobilisation within T cells is seen among $CD8^+$ T cells, with minimal $CD4^+$ T cell mobilisation [47-50]. Although mobilisation of $CD4^+$ T cells is minimal, there is evidence to support that exercise induces a shift from Th1 to Th2 polarization, reducing pro-inflammatory $CD4^+$ T cell phenotypes [6, 7, 79-81]. Within the $CD8^+$ T cell compartment, $CD8^+$ T cells with a highly differentiated memory phenotype are preferentially mobilised following exercise [50-55]. $CD8^+$ EMRA have been reported to increase by 450% following vigorous exercise compared to naïve $CD8^+$ T cells which increased by 84% [51]. Further studies show that $CD8^+$ effector memory (EM) and EMRA that exhibited the largest increase following exercise were fully differentiated ($CD27^-CD28^-$) [52] and expressed markers of terminal differentiation and senescence (killer cell lectin-like receptor subfamily G member 1 $(KLRG1)^+CD57^+$) [53]. Whilst the cells detectable in the T1D pancreas express markers of differentiation, memory and residency [4-7], the effects of acute exercise on the mobilisation of these T cell subsets in T1D is yet to be explored.

Investigating the effects of exercise on T cell subsets in T1D may be critical for the understanding and treatment of this disease. Lymphocytosis is followed by intensity-dependent lymphopenia in the period that follows a bout of exercise [47-49, 56-58]. Following the dramatic increase in $CD8^+$ EM and EMRA frequency following exercise, it is these same subsets which exhibit the largest egress from the peripheral blood during the recovery period [52, 53]. This flux may play a role in immune regulation. Tissue redistribution of lymphocytes is one cause of exercise-induced lymphopenia [58]. Krüger et al. 2007 demonstrated using fluorescent cell tracking in mice that T cells were released from the spleen and accumulated in the lungs, bone marrow, and Peyer's patches of the mice following acute exercise [59]. Such movement of immune cells may support immune surveillance. Post-exercise lymphopenia is also thought to result partially from lymphocyte apoptosis [60-62]. Acute exercise, particularly of vigorous intensity (i.e. above 70% VO_2 max), mobilises $CD95^+$ memory T cells [60, 63]. It has been postulated that CD95 expression may indicate an apoptotic fate and subsequently create "immunological space" [64, 65]. The percentage of apoptotic lymphocytes as well as $CD95^+$ T cells increases following exhaustive exercise (80% VO_2 max) in healthy participants. [60, 62, 63]. Furthermore, mice subjected to a strenuous 90-minute treadmill running protocol showed a decrease in $CD8^+$ T cells 24 hours following exercise, and apoptosis of intestinal $CD8^+$ T cells was higher 24 hours after exercise [61]. It is hypothesised that this vacant immune space following exercise can be taken up by newly generated immature cells, creating the opportunity to reprogram immune memory. In support of this, vigorous acute exercise increases hematopoietic stem and progenitor cells (HSPC) post-exercise as well as stimulates haematopoiesis [66, 67].

Exploring the effects of acute exercise on T cells in T1D

Given that acute exercise mobilises $CD8^+$ T cells with a highly differentiated phenotype that express markers of tissue residency, and that $CD8^+$ T cells with this phenotype are thought to sustain beta cell destruction in T1D, it is important to explore the impact of acute exercise on these T cell subsets in T1D. A limited amount of research has been conducted in exercise training in T1D. Exercise training in streptozotocin-induced T1D mice significantly increased insulin content and insulin secretion compared to sedentary mice [68]. Furthermore, exercise training in non-obese diabetic (NOD) mice reduced immune cell infiltration into the pancreas and subsequently the insulitis index. This is the only exercise study in a model of T1D to demonstrate the modulatory effects of exercise on islet immunity [69]. We therefore aimed to

provide a comprehensive phenotypic characterisation of exercise-induced mobilisation of potentially pathogenic T cell subsets in T1D, and compare this response to that observed in healthy participants.

METHODS

Participants

Ethical approval was granted by the Preston Research Ethics Committee (REC) for this study. Twelve controls and twelve T1D participants were recruited. All participants were male and between 16-65 years of age. Male only participants were chosen to minimise differences in immunity evident in females due to higher oestrogen levels [70-72]. Participant baseline characteristics are reported in table 1. T1D participants had a clinical diagnosis of T1D, were on basal bolus insulin regime or insulin pump therapy, competent in carbohydrate content estimation of meals, were willing to test glucose through capillary testing, and were able to recognise hypoglycaemic symptoms before blood glucose fell to 3.9mmol/L. Participants did not have a history of cardiac disease or other significant illness that would prevent attendance at the study sit. All T1D participants did not have active proliferative diabetic retinopathy, autonomic neuropathy, or history of severe hypoglycaemia requiring third party assistance within the last 3 months prior to the study.

Experimental design

Participants had one enrolment visit, where baseline demographics and anthropometric assessment was carried out (table 1). During the enrolment visit, each participant completed a non-fasted incremental sub-maximal (85% HRmax) cycle ergometer test to calculate their predicted VO_2 max. This was used to calculate workload and heart rate for the subsequent exercise visit adjusted to individual fitness [73]. The enrolment visit and exercise visit were separated by one week. Participants were asked to abstain for vigorous exercise 24 hours prior to the exercise visit. Participants were also required to record a food diary for the 24 hours prior to the exercise visit. Participants were advised to use these diaries to ensure that the same foods were consumed in the 24 hours prior to each exercise bout. . The exercise visit started at 8.30am for all participants and consisted of a thirty-minute bout of cycling at 80% predicted VO_2 max. An initial fasting blood sample was taken for each participant once the cannula was inserted. The participant was then allowed to rest for a further 20 minutes before preparing for the acute exercise bout. Fasting blood samples were collected

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intravenously at immediately pre-exercise, immediately post-exercise, and 1 hour post-exercise. Pre- and post-exercise samples were taken whilst the participant was sitting on the cycle ergometer and sampling was strictly timed using a stopwatch. All participants completed an international physical activity questionnaire (IPAQ) [74] and perceived stress questionnaires; the life scale events questionnaire [75], perceived stress scale [76], the undergraduate stress questionnaire [77], self-perceived health status [78], and the Pittsburgh sleep quality index [79].

Sample processing

All blood samples were processed under identical conditions using the same laboratory reagents and apparatus. Blood samples for immunophenotyping analysis were taken in lithium heparin vacuette tubes (95057-405, Greiner Bio-one GmbH, Frickenhausen, Germany) and placed on roller at room temperature to ensure constant mixing of the blood sample until processing. All sample processing was initiated within 2 hours of blood-draw. Haematological measures were conducted on 25µl of whole blood using an automated coulter counter (ABX Micros ES 60, HORIBA Medical). Relative cell number (cells/µl) of T cell subsets was then calculated from this.

Whole blood staining

The whole blood staining protocol was optimised prior to the start of the study. The protocol was adapted from the Clinical Immunology Service, University of Birmingham. Red blood cells were lysed by preparing whole blood in 4ml aliquots and washed with 16mls Ammonium Chloride lysis buffer (16g Ammonium Chloride (326372, Sigma-Aldrich, Dorset, UK), 2g sodium hydrogen carbonate (S/4240/60, Fisher scientific Ltd, Loughborough, UK), 0.2g EDTA (E5134, Sigma-Aldrich, Dorset, UK), and 2L ddH_2O). The sample was centrifuged at 1000g for 5 minutes. Pelleted cells were resuspended in 10mls RPMI-1640 (R0833, Sigma-Aldrich, Dorset, UK) (supplemented with 2% FBS) and centrifuged at 1000g for 5 minutes. Cells were then counted and resuspended to a concentration of $1x10^6$ cells/ml. Cells were stained with appropriate antibodies and incubated in dark at 4°C for 20minutes. Stained cells were lysed and fixed with 500µl 1X BD FACS lysing solution (containing 14% formaldehyde) (349202, BD Biosciences, Wokingham, UK) and incubated in dark at 4°C for a further 15minutes. Fixed cells were washed (centrifuged at 1000g for 5 minutes) in 2mls phosphate-buffered saline (PBS). Pelleted cells were

resuspended in 500µl PBS and stored at 4°C until flow cytometry analysis. The stability of fixed stains was assessed and confirmed that cells could be stored up to 24hours at 4°C before flow cytometry analysis. All samples were analysed using BD LSR Fortessa X-20. Parent populations (i.e. lymphocytes) were selected based on their size on FSC/SSC dot plots. Doublets were omitted by selecting the linear population shown on FSC-A/FSC-H dot plots prior to recording. Events to record were set to 100,000 within the parent population gate. Compensation was carried out monthly using compensation beads and single stained cells. The most recent compensation set up was linked to each experiment. A negative control (unstained whole blood) was run for each experiment.

T cell subset analysis

Individual T cell phenotypes along the linear differentiation pathway of $CD4^+$ and $CD8^+$ T cells (Figure 1), as well as $CD4^+$ T-helper (Th) and T-Regulatory (TReg) subsets following an acute bout of vigorous intensity exercise (80% predicted VO_2 max) were measured in T1D and healthy participants. Two multi-colour flow cytometry panels were designed to phenotype lineage T cell subsets (panel 1) and CD4 T-helper/T-Regulatory subsets (panel 2) using the following anti-human monoclonal antibodies (mAbs) obtained from BD Biosciences (Wokingham, UK) (unless stated otherwise): ***Panel 1*** anti-CD3 PE-Cy7 (UCHT1), anti-CD4 APC-R700 (RPA-T4), anti-CD8 APC-H7 (SK1), anti-CD11b PE-CF594 (ICRF44), anti-CD27 BB515 (M-T271), anti-CD28 BV510 (CD28.2), anti-CD45RA BV786 (HI100), anti-CD69 BV650 (FN50), anti-CD95 BV421 (DX2), anti-CD127 AF647 (HIL-7R-M21), anti-CCR7 PE (3D12), anti-7-AAD PerCP-Cy5.5. ***Panel 2*** anti-CD3 PE-Cy7 (UCHT1), anti-CD4 APC-R700 (RPA-T4), anti-CD25 PE (M-A251), anti-CD45RO BV786 (UCHL1), anti-CD127 AF647 (HIL-7R-M21), anti-CXCR3 PE-CF594 (1C6), anti-CCR4 BV421 (1G1), anti-CCR6 BV711 (11A9), anti-IL-6R FITC (AS12), anti-TIGIT PerCP-Cy5.5 (MBSA43) (eBioscience, San Diego, CA, US), and Live/Dead-Fixable Viability Stain 780.

Data analysis

Flowjo version 10 (FlowJo LLC, Oregon) was used to analyse flow cytometry data. Doublets were removed using FSC-A versus FSH-H dot plots. Dead cells positive for 7-AAD viability stain were removed, and lymphocytes were selected based on size on SSC-A versus FSC-A dot plots. Total T cells were selected as $CD3^+$, and further selected as separate $CD4^+$ and $CD8^+$ populations. Spider gates were used within $CD4^+$ and $CD8^+$ populations to define

distinct progressively differentiated subsets as outlined in supplementary Figure 1; naïve ($CD45RA^+CCR7^+$), T_{SCM} ($CD45RA^+CCR7^+CD95^+CD127^+$), central memory (CM: $CD45RA^-CCR7^+$), effector memory (EM: $CD45RA^-CCR7^-$), early differentiated (ED: $CD45RA^-CCR7^-CD27^+CD28^+$), early-like differentiated (ELD: $CD45RA^-$ $CCR7^-$ $CD27^-CD28^+$) and intermediately differentiated (ID: $CD45RA^-$ $CCR7^-CD27^+CD28^-$), and effector memory re-expressing CD45RA (EMRA: $CD45RA^+CCR7^-$). The gating strategy for T_{SCM} is displayed in supplementary Figure 2. Single cell surface expression of CD69, CD11b, CD127, and CD95 was gated on CD4 and CD8 naïve, CM, EM, and EMRA T cell subsets.

$CD4^+$ T cells were further divided into T-helper (supplementary Figure 3) and T-Regulatory (supplementary Figure 4) subsets; $CD4^+$ T-Regulatory ($CD127^{lo}CD25^{hi}$), naïve T-Reg ($CD45RO^-CD127^{lo}CD25^{hi}$), memory T-Reg ($CD45RO^-CD127^{lo}CD25^{hi}$), T-helper (Th: $CD127^+CD25^-CD45RO^+$), Th1 ($CXCR3^+CCR6^+CCR4^-$), Th2 ($CXCR3^-CCR6^-CCR4^+$), and Th17 ($CXCR3^-CCR6^+CCR4^+$).

Statistical analysis

Statistical analysis was performed using SPSS version 24 (IBM, Chicago) and GraphPad Prism version 7 (GraphPad Software, California). Firstly, normality tests were performed on all data using Q-Q plots in SPSS. Data which was not normally distributed was logged and normality tests were repeated, confirming all subsets to have normal distribution. Multiple regression analysis was used to analyse within subject's effect (time) and between subject's effects overtime (time*group). Main effects of exercise are described as changes over time. Changes immediately post-exercise and 1 hours post-exercise are compared to baseline values and are reported in tables for each group under the heading "contrast". P values were reported as sphericity assumed however where mauchly's test of sphericity was violated i.e. $p \leq 0.05$, Greenhouse-Geisser corrected value was used. Student T-tests were performed on baseline characteristics. The p values, F values, and degrees of freedom (df) are reported in tables as [F = (df, df error) value, p-value]. Variation in n numbers is a result of a participant having no data for 1 hour post-exercise time-point. Data are presented in tables as mean ± standard deviation (SD) unless otherwise stated. P values ≤ 0.05 were considered significant. Significantly mobilised subsets in control and T1D groups, but blunted in T1D, are presented in tables as bold.

RESULTS

Participants anthropometric and physiologic characteristics are shown in table 1. No statistically significant differences between groups were found for anthropometric and physiologic characteristics. The results obtained for $CD8^+$ lineage subpopulations are summarized in tables 2-3. $CD4^+$ T-Regulatory and T-helper subpopulations are summarized in table 4. Extensive T cell subpopulations analysis is included in supplementary tables 1-3.

The mobilisation $CD8^+$ EMRA T cells is blunted in T1D during vigorous intensity exercise

Lymphocytosis and $CD8^+$ T cell mobilisation occurs in both T1D and control groups as summarized in supplementary table 1. However, the most dramatic changes were observed within the $CD8^+$ lineage subsets (depicted in Figure 1) during vigorous intensity exercise in T1D and control participants. The results for $CD8^+$ lineage subsets are displayed in table 2. As anticipated, within the $CD8^+$ T cell populations, less differentiated cells such as naïve, T_{SCM}, and CM subsets did not significantly change with exercise in either group. As expected, significant changes were however observed in the more differentiated subsets such as $CD8^+$ ED, ELD, ID, EM, and EMRA subsets during vigorous exercise. Therefore, the increase in $CD8^+$ T cells is driven by mobilisation of later differentiated $CD8^+$ T cell subsets, and these changes were most significant in the $CD8^+$ EMRA subsets for each group.

Although statistically significant changes in $CD8^+$ T_{SCM} were not observed during vigorous intensity exercise in either group, increases in the number $CD8^+$ T_{SCM} immediately post-exercise were suggested in both control and T1D participants (table 3). However, during vigorous intensity exercise a blunted response is noted in the T1D group. $CD8^+$ T_{SCM} decreased following vigorous exercise by 90.97% in the T1D group but increased dramatically by 1898.69% in the control group. It is worth noting that the total cell numbers for $CD8^+$ T_{SCM} subsets were very small.

Within the later differentiated subsets, $CD8^+$ ED significantly changed during vigorous intensity exercise overall only ($p=0.040$), but this was not seen in either T1D or control groups independently. There was an overall change in $CD8^+$ ELD during vigorous intensity exercise ($p<0.001$), with a non-significant trend to change in the T1D group ($p=0.054$) but not in the control group. This change was driven by an increase in $CD8^+$ ELD post-exercise followed by a significant decrease below baseline in $CD8^+$ ELD 1 hour post vigorous

exercise in the T1D group only (p=0.029). $CD8^+$ ID significantly changed over time with vigorous exercise (p<0.001) but not in either T1D or control groups independently. $CD8^+$ ID significantly decreased below baseline 1 hour post vigorous exercise in the control group only (p=0.027). $CD8^+$ EM showed a trend to mobilise during vigorous exercise (p=0.067), driven by a trend to increase post-exercise, with a decrease below baseline at 1 hour post-exercise (p=0.040) in the T1D group. No significant changes were observed for $CD8^+$ EM in the control group.

Finally, $CD8^+$ EMRA cell frequency significantly changed overall during vigorous intensity exercise (p=0.001). $CD8^+$ EMRA were significantly mobilised by vigorous intensity exercise, driven by a trend to increase post-exercise, in T1D (p=0.004) and control groups (p=0.010). There was a significant decrease 1 hour post-exercise in both T1D (p=0.019) and control groups (p=0.004). In summary, $CD8^+$ EMRA were the only $CD8^+$ T cells subset mobilised by vigorous intensity exercise in both the T1D and control group. However, the percentage increase post vigorous intensity exercise is much lower in the T1D group (control: 66.67%, T1D: 26.45%) suggesting a blunted egress of $CD8^+$ EMRA during exercise in T1D (Figure 2); however, no significant time*group differences were observed.

Vigorous intensity exercise mobilises fully differentiated $CD8^+$ EMRA T cells expressing markers of activation and adhesion in T1D and control participants, with evidence of a blunted response in T1D

A number of cell surface markers were measured on $CD8^+$ T cells to define the homing propensity and function of circulating $CD8^+$ populations in T1D and control participants. These were examined on naïve, CM, EM, and EMRA $CD8^+$ T cell subsets. These markers included CD69 (a marker of activation and tissue-resident populations), CD11b (an adhesion marker involved in lymphocyte migration), CD127 (IL-7Rα, necessary for memory $CD8^+$ T cell maintenance), and CD95 (expressed on memory subsets and a marker of apoptosis). The combination of CD27 and CD28 expression was used to define the differentiation status of $CD8^+$ EMRA subsets. Conventionally EMRA are defined as $CD27^-CD28^-$. However, more recently further subdivisions of EMRA differentiation based on the differential expression of CD27 and CD28 have been described and also shown in this study herein [14, 15].

As described earlier, $CD8^+$ EMRA are the only subsets which are significantly mobilised in both T1D and control participants. Interestingly, the percentage increase of $CD8^+$ EMRA T

cells is blunted in T1D. Further phenotyping of $CD8^+$ EMRA T cell subsets using the surface markers described above show that $CD8^+$ EMRA expressing markers of activation and tissue residency (CD69), homing propensity (CD11b), and are the most differentiated ($CD27^-CD28^-$) are significantly mobilised by acute vigorous intensity exercise in both groups. Again, the percentage increase of these $CD8^+$ EMRA T cell subsets is blunted in T1D compared to control participants. No significant mobilisation was observed for $CD8^+$ EMRA expressing CD127 or CD95 in either group independently. The results for the above cell surface markers on $CD8^+$ lineage subsets during vigorous intensity exercise in T1D and control participants are displayed in table 3 and described below.

Vigorous intensity exercise significantly mobilised all but naïve subsets expressing CD69. The most profound effect was observed $CD8^+$ EMRA T cells expressing CD69. Vigorous intensity exercise resulted in the mobilisation of $CD8^+CD69^+$ EMRA T cells in T1D ($p=0.002$) and control groups ($p=0.003$), with a significant increase immediately post-exercise observed in the control group only ($p=0.010$). The percentage increase was blunted in the T1D group following vigorous (T1D: 29.87%, Control: 161.43%) intensity exercise (table 3).

Likewise, the most profound effect on $CD8^+$ T cell subsets expressing CD11b was observed for the EMRA. Vigorous intensity exercise significantly mobilised $CD8^+$ EMRA T cells expressing CD11b in T1D ($p=0.007$) and control groups ($p=0.001$), with no significant changes in $CD8^+CD11b^+$ naïve, CM, or EM subsets during vigorous exercise in either group. There was a significant increase immediately post-exercise in the control group only ($p=0.010$). However, in the T1D group, this subset significantly decreased below baseline 1 hour post vigorous exercise ($p=0.006$). The percentage increase of $CD8^+CD11b^+$ EMRA was blunted in the T1D group following vigorous intensity exercise (T1D: 37.70%, Control: 91.48%) (table 3).

Lastly, fully differentiated $CD8^+$ EMRA ($CD27^-CD28^-$) were the only subset of EMRA to significantly mobilise both the T1D ($p=0.050$) and control group ($p=0.037$) independently. The percentage increase post vigorous exercise was considerably blunted in the T1D group (T1D: -7.02%, control: 113.76%). There was a significant decrease below baseline 1 hour post vigorous intensity exercise in the T1D group only ($p=0.005$).

Vigorous intensity exercise mobilises $CD4^+$ T-Regulatory cells, mainly comprised of memory T-Regulatory cells, in control but not T1D participants

Vigorous intensity exercise mobilises $CD4^+$ T cells in control but not T1D participants (supplementary table 1). A similar pattern was observed for $CD4^+$ lineage subsets (supplementary table 2-3). Significant mobilisation patterns were also observed within the $CD4^+$ T-Regulatory and T-helper subsets, and is described below.

Total $CD4^+$ T-Regulatory cells ($CD127^{lo}CD25^{hi}$), and the naïve ($CD45RO^-CD127^{lo}CD25^{hi}$) and memory ($CD45RO^-CD127^{lo}CD25^{hi}$) compartment, were measured during vigorous intensity exercise in T1D and control participants. The results are displayed in table 4. $CD4^+$ TReg subsets were significantly mobilised by vigorous intensity exercise overall (p=0.010) and in control (p=0.009) but not T1D participants. There was a significant decrease below baseline 1 hour post-exercise overall (p=0.038), but this was not seen in either T1D or control groups independently (Figure 3a). The percentage change following exercise was much lower in the T1D compared to the control group (T1D: 23.74%, Control: 47.65%). Further delineation of $CD4^+$ TRegs revealed that memory TReg subsets were significantly mobilised by vigorous intensity exercise overall (p=0.004) and in the control group (p=0.006) (Figure 3b). There was a significant decrease below baseline overall 1 hour post-exercise (p=0.042), but this was not seen in either T1D or control group independently. Naïve TReg subsets did not significantly mobilise (Figure 3c).

Th1 ($CXCR3^+CCR6^+CCR4^-$), Th2 ($CXCR3^-CCR6^-CCR4^+$), and Th17 ($CXCR3^-CCR6^+CCR4^+$) cells were measured during vigorous intensity exercise in T1D and control participants. The results are displayed in table 4. Vigorous intensity exercise mobilised $CD4^+$ Th2 cells in control but not T1D participants. However, neither $CD4^+$ Th1 or Th17 cells significantly mobilised in either control or T1D groups independently. In summary, $CD4^+$ T-helper subsets do not significantly mobilise in T1D participants. However, some mobilisation of Th2 subsets were observed in control participants, shifting the Th1/Th2 ratio towards anti-inflammatory subsets. Th2 subsets significantly mobilised during vigorous intensity exercise in control (p=0.042) but not T1D participants, with a significant increase post-exercise in the control group (p=0.039).

DISCUSSION

This study has, for the first time, characterised the effects of acute exercise on the mobilisation of T cell subsets in people with T1D. The use of surface markers to define T cell function and fate improves understanding of the specific subpopulations mobilised during exercise in a T cell mediated autoimmune disease characterised by profound T cell involvement.

Acute exercise causes an intensity-dependent lymphocytosis in people with T1D. Total lymphocytes, including $CD3^+$ T cells are mobilised in both T1D and control participants. This agrees with previous studies where vigorous exercise induces a significant rise in peripheral blood lymphocytes, followed by lymphopenia, in healthy cohorts [48, 56-58, 80]. Within the T cell compartment, $CD4^+$ T cells mobilised during vigorous exercise in the control group, but not the T1D group. This had a downstream effect on $CD4^+$ T cell subsets in T1D participants because no significant mobilisation of differentiated $CD4^+$ T cell lineage subsets was observed during exercise. In agreement with previous studies, the changes seen during exercise within the $CD4^+$ compartment were minimal compared to changes seen within the $CD8^+$ compartment [50, 51].

Vigorous intensity exercise significantly mobilised $CD8^+$ T cells in both T1D and control participants. $CD8^+$ T cells increased to the same extent after vigorous intensity exercise in both the T1D and control group. As reported in previous studies, $CD8^+$ EMRA were the most sensitive T cell subset to mobilisation, and are shown to mobilise by vigorous exercise in this study herein and others [50-52]. However, the percentage increase of $CD8^+$ EMRA in the T1D group following vigorous intensity exercise was blunted. Further phenotyping of $CD8^+$ EMRA populations revealed that the mobilised subsets mainly comprised of fully differentiated ($CD27^-CD28^-$), recently activated tissue-resident ($CD69^+$) EMRA with migratory capacity ($CD11b^+$). Again, the percentage increase of these $CD8^+$ EMRA subsets following vigorous exercise was blunted in the T1D group. To the best of our knowledge, this is the first time this effect has been described in T1D.

Furthermore, the effects of exercise on T_{SCM} subsets have not been previously examined. This is the first time this has been investigated in T1D and healthy cohorts. Although no significant changes overtime for either $CD4^+$ or $CD8^+$ T_{SCM} were found, a trend to increase following vigorous intensity exercise as shown by percentage increase was observed in healthy participants. Furthermore, an impaired response in T1D was noted for $CD4^+$ and $CD8^+$ T_{SCM} subsets, with both subsets decreasing following vigorous intensity exercise.

In this study, we used additional cell surface markers to define the function and fate of $CD4^+$ and $CD8^+$ T cell lineage subsets. Increases in both CD69 and CD11b on $CD4^+$ and $CD8^+$ lymphocytes have been reported following exercise previously, but not on specific T cell subsets as demonstrated in this study herein [57, 81-89]. An increase in $CD4^+$ and $CD8^+$ T cell lineage subsets expressing CD69 is evident in both groups, with a larger percentage increase post-exercise observed in healthy participants. $CD4^+$ and $CD8^+$ T cell lineage subsets expressing CD11b is also noted following exercise in both groups. However, the percentage increase post-exercise in the T1D participants is smaller. $CD95^+$ memory T cells are known to mobilise following vigorous intensity exercise in healthy participants [60, 63]. In our study, $CD95^+$ EM and EMRA T cells from both the $CD4^+$ and $CD8^+$ compartments increased immediately post-exercise, however this increase did not reach statistical significance. Again, it is evident that this response is blunted in T1D as the percentage increase following vigorous intensity exercise is much lower than that of the control group.

There are a number of potential reasons for this blunted response in T1D. Firstly, blood glucose levels are elevated in people with T1D (above 5mM) during and following. High glucose conditions affect the mobilisation of several cell types and therefore may affect lymphocytosis. Elevated plasma glucose in T1D and T2D rodent models have been reported to impair $CD34^+$ HSPC, $CD45^+$, and fibroblast cell migration [90, 91]. Furthermore, fewer lymphocytes were found in the wound site of streptozotocin-induced diabetic mice compared to control, suggesting lower migration of lymphocytes in T1D [92]. Therefore, high glucose levels in T1D may have an effect on T cell migration during exercise.

Secondly, the T cell subsets which exhibit blunted lymphocytosis during vigorous exercise in T1D are those which typically exhibit a high level of beta-adrenergic receptor expression. It is recognised that catecholamine responses can be blunted in T1D. Natural killer (NK) cells and cytotoxic T lymphocytes express much higher levels of beta-adrenergic receptors than other mononuclear cells, causing their dramatic mobilisation during exercise [93-96]. Alterations in these receptors in T1D would alter the stress response to exercise. Reduced beta-adrenergic sensitivity of lymphocytes in T1D has been reported, resulting in a dampened adrenaline response [97-99]. During acute exercise, increased beta-adrenoceptor density and sensitivity of lymphocytes is noted in healthy participants. However, patients with congestive heart failure (CHF) exhibited a blunted increase in beta-adrenoceptor density and no increase in sensitivity [100]. A similar effect may be seen in T1D and may impact exercise-induced lymphocytosis.

Lastly, the T cell subsets exhibiting blunted lymphocytosis during vigorous exercise share characteristics with those sequestered in the T1D pancreas [6, 7, 101, 102]. These include $CD8^+CD69^+$ T cells [101, 103], $CD8^+CD11b^+$ T cells [6, 7], and highly differentiated memory T cells [2, 4, 8]. The proportion of $CD8^+CD69^+$ T cells was higher in the pancreas than salivary glands taken from NOD mice [101]. $CD8^+CD11b^+$ T cells have also been found within NOD islets [6, 7] and found to be higher in the islets of NOD mice compared to peripheral blood [102]. Furthermore, antigen-experienced islet reactive $CD8^+$ T cells were found sequestered in the pancreas of T1D donors [4]. Islet reactive $CD8^+$ T cells have been shown to exhibit a highly differentiated memory phenotype [2, 8], similar to the $CD8^+$ EMRA T cell phenotype. More recently, islet specific $CD8^+$ T cells were shown to display a T_{SCM} phenotype. In our study, lymphocytosis of highly differentiated $CD8^+$ EMRA that express CD69 and CD11b was blunted immediately following vigorous exercise in T1D. Both $CD4^+$ and $CD8^+$ T_{SCM} also displayed a blunted percentage increase following vigorous exercise compared to the control group. It therefore is possible to postulate that the blunted increase following exercise in these $CD8^+$ T cell subsets is due to their sequestration in the pancreas in T1D.

The results of our study have a number of implications for T1D. Firstly, this study provides imperative evidence of the effects of acute exercise on immunity in people with T1D. Importantly, this provides a platform for future investigations of exercise in T1D, allowing for new avenues to be explored to increase the initial honeymoon phase following diagnosis [104], reduce disease severity, and ultimately for the treatment of T1D. Acute exercise has the potential to regulate immunity in T1D through increased immunosurveillance, deletion of islet reactive T cells and thereby creation of immune space. Lymphocyte trafficking and tissue redistribution is essential for immunosurveillance and regulation [58, 59]. The deletion of exercise sensitive EM and EMRA $CD8^+$ T cells following acute exercise has the potential to regulate immunity through the creation of "immunological space" in people with T1D [60-65]. This immune space following exercise could be taken up by newly generated HSPC and reprogram immune memory [66, 67], thereby reducing aggressive memory T cell phenotypes and ultimately modulating beta cell autoimmunity in T1D.

Furthermore, acute exercise could be used in combination with current trial immunotherapies, and possibly boost the response to immunotherapeutic agents, which to date have been lacking for treatment of T1D. Some immunotherapeutic approaches to modulate beta cell autoimmunity in T1D aim to reduce aggressive memory phenotypes and promote the

generation of new naïve cells in T1D. This has been achieved previously with both acute exercise and exercise training in healthy cohorts. Therefore, exercise in T1D may be used as an adjunct for other immunotherapeutic agents. One such immunotherapy, teplizumab, a nonactivating anti-CD3 monoclonal antibody, aims to reduce effector memory subsets, and increase naïve and early memory subsets. This could potentially also be achieved solely by an acute exercise bout or used to boost target T cell populations by administering the treatment directly after an acute exercise bout. Anti-CD3 mAbs that are non-Fc receptor (FcR) binding, like teplizumab, selectively induce apoptosis of antigen-activated T cell phenotypes such as those with memory/pathogenic phenotypes but not naïve T cells [105]. Teplizumab treatment resulted in maintained or improved beta cell function for at least 2 years post treatment in recent-onset T1D patients [106-109]. Evidence for beta cell preservation was also reported in long standing T1D patients up to 1 year post clinical diagnosis [110]. One study identified a group of responders who had higher activated $CD8^+$ terminally differentiated effector and $CD8^+$ EM in T cells at baseline [111]. This is consistent with increased $CD8^+$ terminally differentiated EM T cells following acute exercise. Therefore, frequent bouts of acute exercise could create an environment in which T1D participants respond better to treatment.

Preliminary data from this study herein also provides a basis to investigate exercise training (as opposed to a single bout of exercise) in T1D. Chronic exercise reduces senescent T cells in the blood [112]. In an ageing study, physically fit age matched controls had a lower proportion of peripheral blood memory T cells ($KLRG1^+CD57^+$, $KLRG1^+CD28^-$) compared to those with a lower VO_2 max [65, 113]. Naïve-memory T cell balance is disrupted in the ageing population and a similar observation has been found in T1D [114]. Therefore, reduced senescent memory T cell populations may also occur from exercise training in T1D.

The mechanisms by which exercise training could preserve beta cells in T1D have yet to be explored. Evidence of immunomodulation by exercise training in T1D is limited. Two studies to date have examined exercise training in T1D mouse models. The first study showed that 6 weeks of exercise training in streptozotocin-induced T1D mice significantly improved insulin content and insulin secretion in islets compared to sedentary mice, suggesting a protective effect against the destruction of the remaining beta cells or the generation of new beta cells [68]. A more recent study has examined the effects of exercise training on immune parameters in T1D [69]. Twenty weeks of training in non-obese diabetic (NOD) mice resulted in reduced immune cell infiltration into the pancreas and subsequently the insulitis

index. This is the only exercise study in a model of T1D to demonstrate the modulatory effects of exercise on islet immunity [69].

Although not well studied in T1D, exercise training has been demonstrated to modulate immunity in other T cell mediated autoimmune disorders [115]. Mice with EAE, a model for multiple sclerosis (MS), underwent 6 weeks exercise training. T cells taken from lymph nodes had an inhibited immune response to autoantigen whilst sustaining an increased immune response to non-specific stimulus such as concanavalin A [115]. This suggests the generation of new naïve and early memory T cells with exercise training as the recall response to autoantigen is reduced. Another study showed improved immune modulation by exercise in EAE models resulting in delayed onset of disease and increased T cells with a regulatory phenotype [116]. However, other autoimmune disorders such as rheumatoid arthritis (RA) and systemic lupus (SLE) have different responses to exercise, where $CD8^+$ T cells are reduced following exercise and $CD4^+$ T cells are reduced at peak exercise but increased after cessation [117]. It is unclear if exercise training (as opposed to a single bout of exercise) would result in responses to exercise that are comparable to healthy participants.

In conclusion, we show for the first time that acute exercise preferentially mobilises differentiated and antigen experienced $CD8^+$ T cells in T1D, but to a lesser extent than in healthy individuals. A contributing factor to the relatively reduced mobilisation pattern in T1D was attributable to a blunted response among highly differentiated $CD8^+$ T cells, which may indicate sequestering of $CD8^+$ T cells in the pancreas. These findings need to be extended and investigated in an exercise training programme, and the functional implications of this effect on beta cell function explored in a formal clinical trial.

We have previously hypothesised that an exercise training programme has the potential to modulate beta cell loss in people newly diagnosed with T1D [118]. We have tested this hypothesis in a pilot randomised controlled trial [119, 120]. This study showed that beta cell function, when corrected for the changes in insulin sensitivity that accompany physical exercise, appears to be preserved in people with T1D. The results of this most recent work exploring the effect of a single bout of exercise on T cell mobilisation provides mechanistic insight into how exercise may bring about a benefit to beta cell health in people newly diagnosed with T1D. Further evaluation of the immunomodulatory effects of acute exercise on autoreactive memory T cells in T1D is warranted to ascertain impact on disease prognosis. These findings need to be validated in an exercise training study.

Abbreviations

BMI	Body Mass Index
BP	Blood Pressure
CA^{2+}	Calcium
CCL	Chemokine Ligand
CCR	Chemokine Receptor
CD	Cluster of Differentiation
CMV	Cytomegalovirus
CTL	Cytotoxic T Lymphocyte
CXCR	C-X-C chemokine receptor
ddH_2O	Double-distilled water
ED	Early Differentiated
EDTA	Ethylenediaminetetraacetic Acid
ELD	Early-like Differentiated
EM	Effector Memory
EMRA	Effector Memory re-expressing CD45RA
EXTOD	Exercise for Type One Diabetes
FBS	Fetal Bovine Serum
FcR	Fc Receptor
FMO	Fluorescence Minus One
FoxP3	Forkhead winged helix transcription factor
FSC-A	Forward Scatter-Area
FSC-H	Forward Scatter-Height
HbA1c	Haemoglobin A1c
HSPC	Hematopoietic Stem and Progenitor Cells
ID	Intermediately Differentiated
KLRG1	Killer cell Lectin-like Receptor subfamily G member 1
mAb	Monoclonal antibody
mTReg	memory T-Regulatory
naTReg	Naïve T-Regulatory
NK	Natural Killer
O2	Oxygen
PBS	Phosphate Buffer Saline
pTReg	periphery T-Regulatory cell
REC	Research Ethics Committee
S1PR	Sphingosine-1-Phosphate Receptor
sIL2RA	soluble IL2RA
sIL-6R	soluble IL-6R
SSC-A	Side Scatter- Area
SSC-H	Side Scatter- Height
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes

Th1	Type 1 helper
Th2	Type 2 helper
Th17	Type 17 helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TReg	T-Regulatory
T_{SCM}	Stem cell like memory T cells
UHBFT	University Hospitals Birmingham NHS Foundation Trust
WTCRF	Wellcome Trust Clinical Research Facility
ZnT8	Zinc Transporter

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